SYNERGISTIC INTERACTION BETWEEN POTATO GLYCOALKALOIDS α-SOLANINE AND α-CHACONINE IN RELATION TO DESTABILIZATION OF CELL MEMBRANES: ECOLOGICAL IMPLICATIONS

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Abstract—In studies of the lysis of rabbit erythrocytes, red beet cells, and *Penicillium notatum* protoplasts by the potato glycoalkaloids α-solanine and α-chaconine, the latter was consistently the more membrane-disruptive compound and erythrocytes the more susceptible cell type. A 1:1 mixture of solanine and chaconine produced pronounced synergistic effects in all three test systems. In beet cells, such effects were apparent from an early stage of treatment and persisted over a period of several hours. With erythrocytes and fungal protoplasts, the synergism was maximal with mixtures containing approximately 70% chaconine, whereas with beet cells it peaked at approximately 40% chaconine. Synergistic interactions between solanine and chaconine also occurred with regard to cholesterol binding in vitro, with a maximum response corresponding to the 50% mixture. The implications of these findings for the nature and efficacy of chemical defense systems in plants are discussed.

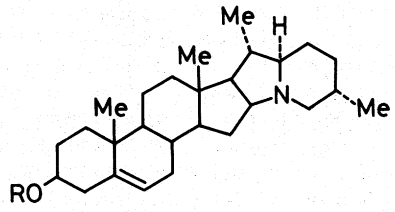
Key Words—Potato, *Solanum tuberosum*, glycoalkaloids, α-solanine, α-chaconine, cell membrane disruption, in vitro sterol binding, synergistic interactions.

INTRODUCTION

It is now generally accepted that plants are able to withstand herbivores and microbial pathogens largely because they have evolved effective chemical

defense systems (Wallace and Mansell, 1976; Harborne, 1978; Rosenthal and Janzen, 1979; Cooper-Driver et al., 1985). However, relatively few detailed biochemical investigations of such defenses have been carried out compared with the vast range of secondary compounds elaborated by plants (Bell and Charlwood, 1980). Of the studies that have been reported, the majority have been concerned with single compounds or single classes of compounds. This approach has yielded interesting and valuable information but suffers from two major limitations: (1) it fails to acknowledge that the behavior and physiology of feeding herbivores or invading microorganisms are often based on contact with a wide diversity of plant chemicals rather than a single compound, and (2) within such a "multichemical" defense system, certain compounds may interact to produce effects qualitatively or quantitatively different from those predictable from the effects of individual compounds.

Much work on chemical defense in the potato plant (*Solanum tuberosum* L.) has centered on the two major steroidal glycoalkaloids, α-solanine and α-chaconine (Figure 1). These compounds, which share a common aglycone (solanidine) and differ only in their carbohydrate moiety, have powerful physiological and pharmacological activity (Roddick, 1986) that has resulted in numerous human and livestock poisonings and fatalities (Morris and Lee, 1984). In addition, there is evidence that these glycoalkaloids, due to their toxicity and/or bitterness, play some role as resistance factors against insects (Tingey, 1984) and, possibly to a lesser extent, as antifungal compounds (Roddick, 1987). In such organisms, deterrence by glycoalkaloids is attributable in large measure to the membrane destabilizing properties of these compounds (Mitchell and



Solanidine, R = H

α-Solanine, R = glu–gal–
rham

α-Chaconine, R = rham–glu–
rham

FIG. 1. Structures of potato glycoalkaloids.

Harrison, 1985; Roddick, 1987). A number of studies in which single glycoalkaloids were tested on a wide variety of organisms from viruses to mammals (e.g., Thorne et al., 1985; Sharma et al., 1979) consistently showed chaconine to be the more potent of the two compounds. We observed similar differential activity with regard to lysis of phosphatidylcholine–sterol liposomes (Roddick and Rijnenberg, 1986) and, more recently, have demonstrated that, in combination, solanine and chaconine produce a pronounced and highly reproducible synergistic effect (Roddick and Rijnenberg, 1987). The synergism was quite specific and did not occur if one of the glycoalkaloids was substituted by related compounds, e.g., tomatine, digitonin, or β_2-chaconine. However, because this study employed synthetic lipid membranes, the biological (and particularly ecological) implications of the synergism cannot be properly assessed. Work in this field has therefore been extended to include the responses of membranes of living cells, and in this communication we report the effects of solanine and chaconine, both singly and in combination, on the integrity of representative cells from animals, plants, and fungi.

METHODS AND MATERIALS

Source of Glycoalkaloids. Solanine and chaconine were extracted from *S. tuberosum* cv. Wauseon and purified by the method of Swain et al. (1978). Authentic samples were also purchased from Sigma Chemical Company, Poole, U.K.

Preparation and Treatment of Erythrocytes. Rabbit blood was collected into heparinized tubes and used either fresh or after storage at 4°C for up to two days. The cells were washed twice with 9× the blood volume of phosphate-buffered saline, pH 7.4, with centrifugation at 400*g* for 10 min after each wash. The final erythrocyte pellet was resuspended in its original volume of buffered saline, and a portion of this suspension removed and diluted to 2% with buffered saline. A 3-ml aliquot of the 2% suspension was incubated with 1 ml of glycoalkaloid solution in buffered saline at 37°C for 2 hr. Glycoalkaloid solutions were prepared by dissolving in the minimum volume of 0.1 M HCl and making up to volume with buffered saline. Erythrocytes were counted using a hemacytometer and the percentage of lysed cells calculated by comparing with counts in the control containing buffered saline only. In solanine–chaconine interaction experiments, the two glycoalkaloids were prepared as 50 μM solutions and mixed in the ratios indicated. The total alkaloid concentration in all test mixtures was 12.5 μM.

Preparation and Treatment of Beet Disks. Cylinders (1 cm diam.) of the swollen tap root of red beet (*Beta vulgaris* L.) were removed with a cork borer and cut into disks 2 mm thick. These were washed in running tap water for 16

hr and lightly blotted to remove excess water. Ten disks were placed in a 50-ml conical flask along with 10 ml of test solution. Glycoalkaloid solutions were prepared by dissolving in acid as above and making up to volume with phosphate–citrate buffer, pH 7.2. Flasks were closed with cotton wool plugs and placed on a reciprocating shaker at 100 rpm. Mixtures were incubated at 25°C, and at various times the test solutions were decanted and their absorbance at 535 nm measured to quantify betanin loss and, where appropriate, returned to the flask. In interaction experiments, 50 μM solutions of solanine and chaconine were used and mixed in the ratios indicated. The total alkaloid concentration in flasks was 50 μM.

Preparation and Treatment of Fungal Protoplasts. The fungal culture used was *Penicillium notatum* Westling, IMI 17968, maintained on a 2% agar-solidified medium containing 2% malt agar at 20°C. Protoplasts were prepared by a method based on that of Hamlyn et al. (1981). Liquid cultures were initiated by inoculating a medium consisting of 2% sucrose, 0.6% $NaNO_3$, 0.15% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.5% trace element solution (42 mg/liter $CuCl_2 \cdot 2H_2O$, 317 mg/liter $ZnSO_4 \cdot 7H_2O$, 15 mg/liter $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 32 mg/liter $MnSO_4 \cdot 4H_2O$, 398 mg/liter $FeSO_4 \cdot 7H_2O$, and 400 mg/liter EDTA) with a spore suspension in sterile distilled water to give a final spore load of 10^6/ml of medium. The culture was incubated at 25°C for 63 hr, after which the fermenter (21) contents were vacuum-filtered through fine nylon (mesh size approx. 100 μm) which retained mycelium but not spores or small mycelial fragments. The mycelial mat was washed with 20 ml 100 mM MES–NaOH buffer, pH 5.8, containing 0.8 M KCl, after which 5 g of mycelium was removed and resuspended in 50 ml of the same buffer. To 7.5 ml of this suspension in a beaker was added 2.5 ml of a 2.4% (w/v) solution of Novozym 234 (Novo Bio Labs, Bagsvaerd, Denmark) in 100 mM MES–NaOH buffer. The yield of protoplasts was adjusted by altering the amounts of mycelium and Novozym used, but the ratio of the two components was maintained as given above. The mixture was stirred gently at 25°C, and the extent of protoplast formation was monitored by hourly microscopic examinations. After 3 hr of incubation, the digest was gravity-filtered through single muslin and the protoplasts concentrated by centrifugation of the filtrate at 3000*g* for 5 min. The pellet was resuspended in 20 ml of 20 mM MES–NaOH buffer containing 0.8 M KCl and then filtered through double muslin. The filtrate was centrifuged at 1200*g* for 5 min and the supernatant discarded. The protoplast pellet was resuspended in 7.5 ml of 50 mM potassium hydrogen phthalate containing 1.2 M sorbitol with the pH adjusted to 7.2 with tetramethylammonium hydroxide. This suspension was subjected to a final filtration through single muslin before use. Protoplasts were treated with glycoalkaloids by adding 462.5 μl of suspension to 37.5 μl of alkaloid solution in 0.002 M HCl in a 5-ml specimen tube. Reaction mixtures were left at 25°C for 1 hr, after which an aliquot was removed and protoplast

number determined using a hemacytometer. The percentage of protoplasts lysed was calculated by comparing numbers in test samples and controls containing acid only. In interaction experiments, stock solutions of 0.67 mM alkaloid were used to prepare solanine–chaconine mixtures. The total alkaloid concentration in all treatments was 50 μM.

Glycoalkaloid–Sterol Complex Formation In Vitro. The extent of complex formation was determined by measuring the amount of unbound sterol remaining in solution. Glycoalkaloids and cholesterol were dissolved in 96% (v/v) ethanol at strengths of 1 mM and 20 mM, respectively. Solutions were mixed in the ratio of 2.0 ml : 0.1 ml in 15 ml polypropylene tubes, heated at 90°C for 5 min and cooled at room temperature for 1 hr and at 4°C overnight (16 hr). Tubes were centrifuged at 27,000*g* for 30 min at 4°C and the supernatant decanted. A 0.5-ml aliquot was transferred to a 5-ml vial and the solvent evaporated in a nitrogen stream at room temperature. The residue was dissolved in 0.25 ml of ethyl acetate containing 0.04% (w/v) 5α-cholestane. The cholesterol concentration of this solution was determined by GLC for which 5α-cholestane acted as internal standard. A 1-μl sample was injected into a 2-m × 6-mm glass column packed with 3% OV-101 on Gas Chrom Q. The nitrogen flow rate was 50 ml/min and the column and detector (flame ionization) temperatures 275 and 325°C, respectively. Cholesterol was quantified using a precalibrated peak integrator.

Experimental Procedure. All treatments were replicated as indicated in the text, and each experiment was carried out at least twice. Data were analysed using a *t* test and by calculation of least significant differences (LSD) at $P = 0.05$.

RESULTS

As a prerequisite to investigating the interaction between solanine and chaconine, the lytic action of the individual glycoalkaloids against mammalian erythrocytes, red beet cells, and *P. notatum* protoplasts was examined. Dose–response curves are shown in Figure 2.

The greater membrane-lytic effect of chaconine was confirmed in all three systems. Rabbit erythrocytes proved the most susceptible to chaconine, showing lysis at around 1 μM (Figure 2a), whereas beet and protoplasts were unaffected by concentrations less than 10 μM (Figures 2b and c). Similarly, a maximal effect on blood was achieved with 25 μM chaconine (Figure 2a), whereas beet and protoplasts required around 60 μM and 100 μM, respectively (Figures 2b and c). Erythrocytes and beet showed a similar reduced sensitivity to solanine, lysis being first observed at 15–20 μM (Figures 2a and b). However, whereas 100 μM solanine caused almost 100% hemolysis, it elicited only

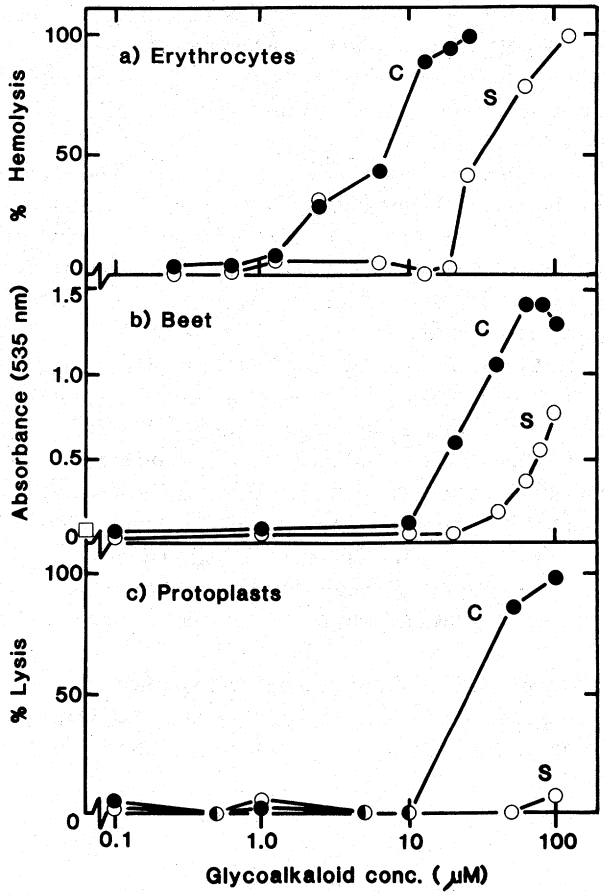


FIG. 2. Semilog plot showing effects of solanine and chaconine on disruption of (a) erythrocytes, (b) beet root cells, and (c) *P. notatum* protoplasts. Cells were treated in buffered solutions, pH 7.2–7.4, for 2, 4, and 1 hr, respectively. Lysis was assessed in erythrocytes and protoplasts by hemacytometer counts relative to controls and in beet cells by measuring the absorbance of the bathing solution at 535 nm. Each point is the mean of three replicates (five counts per replicate) for erythrocytes and five replicates for beet and protoplasts. S = solanine; C = chaconine.

40% loss of betanin from beet cells (Figures 2a and b). In contrast, fungal protoplasts were virtually unaffected by solanine concentrations up to 100 μM (Figure 2c).

From these data, we decided to explore the combined effects of solanine and chaconine in a 1:1 ratio using each compound at 6.25 μM against eryth-

rocytes and 25 μM against beet and protoplasts. At these levels, solanine did not lyse any of the cells and chaconine effects (where present) were less than maximal, thus facilitating the detection of any additive or synergistic responses. In all three test materials, the solanine–chaconine combination gave rise to pronounced synergisms (Table 1). In fact, with beet, the glycoalkaloid mixture was more disruptive than full-strength chaconine. With erythrocytes and *Penicillium* protoplasts, however, the mixture, while synergistic, was less so and approximately the same as full-strength chaconine. A time-course study on beet indicated that the synergism occurred from the outset of the experiment and persisted over a period of several hours (Figure 3). The actual magnitude of the synergism increased for the first 3 hr, after which it remained relatively constant.

The fact that potato tissues do not always accumulate solanine and chaconine in a 1:1 ratio (chaconine is usually more abundant than solanine) prompted us to examine the effects of other glycoalkaloid combinations ranging from 100% solanine to 100% chaconine in steps of 10% (Figure 4). The most hemolytic glycoalkaloid mixture comprised approximately 70% chaconine and 30% solanine, with some indication of a slight supraoptimal effect at higher chaconine levels (Figure 4a). The curve joining the values corresponding to zero, half-strength (6.25 μM), and full-strength (12.5 μM) chaconine only, confirms that the data in Figure 4a represent a series of real synergistic effects and not a dose–response curve for chaconine. Consistent with the results in Table 1, the 50% alkaloid mixture was less hemolytic than 100% chaconine (Figure 4a). In beet tissue, stronger synergisms were observed than in erythrocytes,

TABLE 1. EFFECT OF SOLANINE AND CHACONINE AND THEIR INTERACTION ON DISRUPTION OF ERYTHROCYTES, BEET ROOT CELLS, AND *P. notatum* PROTOPLASTS

Treatment[a]	Hemolysis (%)	A_{535}	Protoplast lysis (%)
Control	—	0.03 ± 0.007	—
Solanine (FS)	11.4 ± 5.59	0.11 ± 0.009	17.9 ± 7.31
Chaconine (FS)	89.0 ± 0.32	0.38 ± 0.080	70.9 ± 4.75
Solanine (HS)	3.2 ± 1.64	0.03 ± 0.005	2.3 ± 1.41
Chaconine (HS)	51.6 ± 3.56	0.15 ± 0.019	4.5 ± 1.03
Solanine (HS) + chaconine (HS)	70.6 ± 2.60*	0.88 ± 0.061***	73.8 ± 1.53***

[a]FS = full strength = 12.5 μM for erythrocytes, 50 μM for beet and protoplasts; HS = half strength. Cells were treated in buffered solutions, pH 7.2–7.4, for 2, 4, and 1 hr, respectively. Lysis was assessed in erythrocytes and protoplasts by hemacytometer counts relative to controls and in beet cells by measuring the absorbance of the bathing solution at 535 nm. Each value represents the mean of five replicate determinations ± standard error. *, *** = Significantly different from Σ solanine (HS) + chaconine (HS) (all combinations) at 5%, 0.1% level.

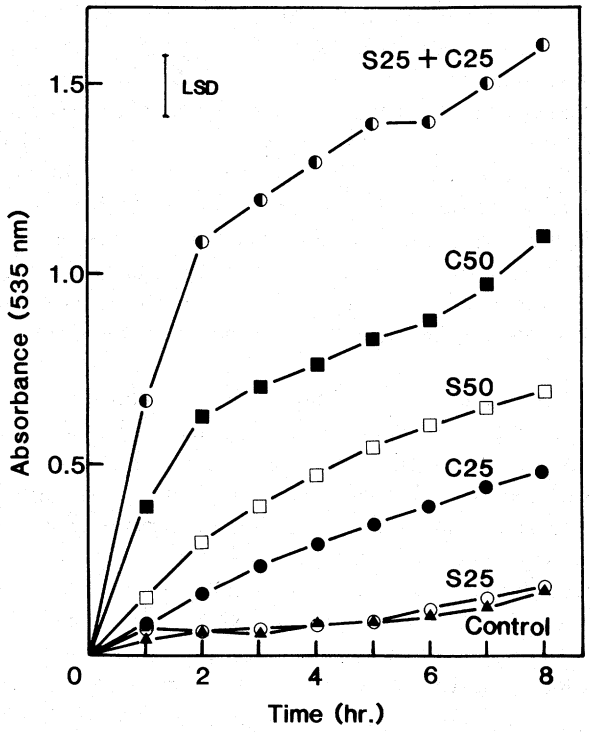


FIG. 3. Effects of solanine and chaconine and their interaction on leakage of betanin from root cells of red beet over a period of time. At various intervals, incubation solutions were decanted, their absorbance at 535 nm measured and returned to flasks. Each point is the mean of five replicates. S = solanine; C = chaconine. Values following abbreviations represent concentration in μM.

with a marked peak response corresponding to approximately 40% chaconine (Figure 4b). The earlier observation that a 50% glycoalkaloid mixture was significantly more disruptive than 100% chaconine (Table 1) was again borne out in this experiment (Figure 4b). The synergistic effects of the solanine–chaconine mixtures on *Penicillium* protoplasts (Figure 4c) bore more resemblance to those on erythrocytes than on beet, with a maximal response again at around 70% chaconine. However, in this case, the 50% mixture and 100% chaconine were of similar efficacy, an observation in keeping with the data in Table 1.

Since membrane destabilization by glycoalkaloids is thought to result from their complexing with membrane sterols (Arneson and Durbin, 1968; Roddick and Drysdale, 1984), experiments were conducted to determine whether solan-

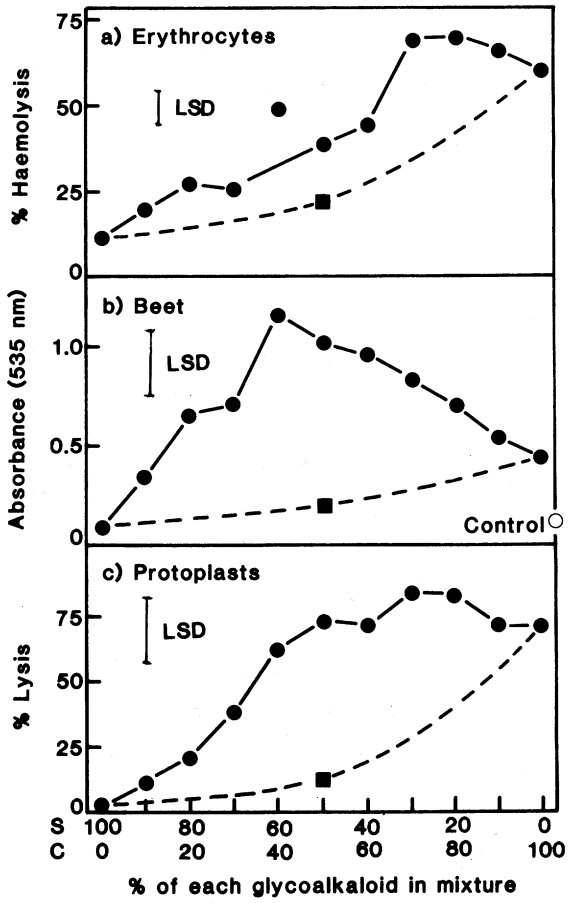


FIG. 4. Effect of relative amounts of solanine and chaconine on disruption of (a) erythrocytes, (b) beet root cells, and (c) *P. notatum* protoplasts. The total glycoalkaloid concentration was 12.5 μM for erythrocytes and 50 μM for beet and protoplasts distributed between solanine and chaconine as shown. Treatments, assessment of cell disruption, and replication were as in Figure 2. ■ = 6.25 μM chaconine only in (a), 25 μM chaconine only in (b) and (c); S = solanine; C = chaconine.

ine and chaconine also interacted synergistically in relation to cholesterol binding in vitro. Using similar glycoalkaloid mixtures, synergistic responses were observed which peaked with the 50% solanine–chaconine mixture (Figure 5). As previously (Figure 4), synergisms are particularly apparent when data are compared with those for single alkaloids (Figure 5, broken lines). In contrast

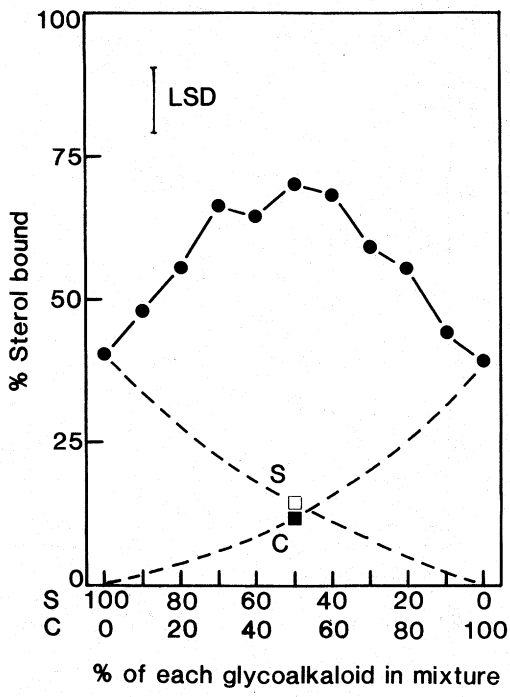


FIG. 5. Effect of relative amounts of solanine and chaconine on glycoalkaloid binding to cholesterol in vitro. The total glycoalkaloid concentration was 1 mM distributed between solanine and chaconine as shown. Reactants were prepared in 96% ethanol and incubated for 1 hr. Cholesterol binding was determined by measuring unbound sterol by GLC relative to the control. Each point is the mean of five replicates. ■ = 500 μM chaconine only; □ = 500 μM solanine only; S = solanine; C = chaconine.

to their differential lytic effects, both solanine and chaconine showed a similar affinity for cholesterol in vitro, an observation which is consistent with earlier reports (Roddick and Rijnenberg, 1986).

DISCUSSION

The greater lytic effect of chaconine than solanine on erythrocytes, beet cells, and *Penicillium* protoplasts is in keeping with numerous reports of similar differential activity against *Herpes simplex* (Thorne et al., 1985), *Alternaria solani* (Sinden et al., 1973), *Fusarium caeruleum* (McKee, 1959), erythrocytes (Tschesche and Wulff, 1964), nematodes (Allen and Feldmesser, 1971), Colorado potato beetle (Mitchell and Harrison, 1985), mice (Sharma et al., 1979) and liposomes (Roddick and Rijnenberg, 1986) and also in taste tests (Zitnak

and Filadelfi, 1985). Total lack of activity of solanine, as found here against *Penicillium* protoplasts, has also been observed with *Herpes* virus (Thorne et al., 1985) and liposomes (Roddick and Rijnenberg, 1986). The carbohydrate moiety is thus an important determining factor in the biological activity of these compounds, although no biochemical explanation of this phenomenon yet exists. The possibility that this effect might be explained by solanine and chaconine differing in their response to degradative enzymes and/or in their ability to reach binding sites in membranes cannot be ruled out.

The synergism between solanine and chaconine previously observed in relation to destabilization of synthetic phosphatidylcholine–sterol liposomes (Roddick and Rijnenberg, 1987) is now also shown to hold for membranes of living cells from taxonomically diverse groups. Further, the synergisms caused by different solanine–chaconine mixtures are not a simple reflection of the differential lytic ability of the two compounds but show an optimum response at particular ratios. The reasons for this remain obscure, but it is probably significant that the mixtures of solanine and chaconine usually found in potato tissues (60–70% chaconine in tubers; Fitzpatrick et al., 1977; Bushway et al., 1980; 70–80% chaconine in shoots; Ahmed and Müller, 1979; Gregory et al., 1981) are those apparently most effective against animal and fungal membranes. However, whether solanine and chaconine interact synergistically against animals and fungi is not yet known.

Data for in vitro cholesterol binding by different solanine–chaconine mixtures correlate well with those for lysis of beet cells (correlation coefficient $r = 0.77$, $P < 0.01$). On the other hand, with erythrocytes and *Penicillium* protoplasts, cell disruption remained high at higher levels of chaconine, while sterol binding fell away, and no significant correlations were observed between sterol binding and cell disruption ($r = 0.008$ and 0.35, respectively). Although cholesterol is a minor sterol in plants and fungi, the major sterols of these organisms, i.e., sitosterol and ergosterol, complex with potato glycoalkaloids in vitro to a similar extent (Roddick and Rijnenberg, 1986). Consequently, it remains open to question whether the solanine–chaconine synergism is due to sterol binding, whether it is achieved through some other mechanism (e.g., penetration into the membrane), or whether different mechanisms operate in different types of membranes. We have previously expressed reservations concerning the relevance of in vitro sterol binding data to in vivo cell toxicity (Roddick and Drysdale, 1984; Roddick and Rijnenberg, 1986), and similar caution probably needs to be exercised in this case.

Although under natural conditions potato glycoalkaloids encounter erythrocytes only rarely (Nishie et al., 1971) and *Penicillium* protoplasts and beet cells not at all, the responsiveness of these cells suggests that the synergism between solanine and chaconine in relation to membrane disruption is a general biological phenomenon rather than a rare event restricted to particular types of

cells. In addition to their lytic properties, solanine and chaconine may protect plants through other effects, e.g., taste (Zitnak and Filadelfi, 1985) and anticholinesterase activity (Orgell, 1963; Alozie et al., 1978), but whether these parameters are affected synergistically by the two compounds remains to be established.

To date, most studies on the biological activity of potato glycoalkaloids have employed single, pure compounds, but our demonstration of a synergistic interaction between solanine and chaconine questions the validity of extrapolating from single compound effects to in situ interactions between potato tissues and other living systems. In addition, the possibility that solanine and chaconine might further interact with other potato compounds cannot be ruled out. Our findings also raise the question of the extent to which synergisms might occur between other glycoalkaloids, e.g., between solasonine and solamargine, which share a common aglycone (solasodine) and possess the same carbohydrate moieties as solanine (solatriose) and chaconine (chacotriose), respectively, as well as between other quite different secondary plant compounds.

The idea that plant allelochemicals may function in an interactive capacity is a relatively recent one. Commercial insecticide synergists have been known for some time (Metcalf, 1967) but few biochemical/ecological studies of naturally occurring compounds have been conducted in this area. Berenbaum (1985) demonstrated a synergistic interaction between myristicin and xanthotoxin with regard to their deleterious effect on larvae of *Heliothis zea*, while Miyakado et al. (1983) found that two amides of *Piper nigrum*, pellitorine and piperine, although individually nontoxic to *Callosobruchus chinensis*, were highly so in a 1 : 1 combination. Piperine and myristicin (also nontoxic) apparently enhance toxicity by interfering with the activity of detoxifying multifunction oxidase enzymes and are therefore referred to as "quasi" synergists (Berenbaum, 1985). Saponins may also function in a quasisynergistic capacity by affecting the penetration, transport, etc., of biologically active molecules (Freeland et al., 1985). In contrast, the synergism between solanine and chaconine is more an "analog" type (Berenbaum, 1985) where two chemically related compounds of differing biological activity interact such that the efficacy of one or both is increased. However, it is not known if chaconine increases the activity of solanine, or vice versa, or both. Structural analogs are widespread within many groups of secondary plant compounds and may have been selected for partly because chemical defense systems incorporating synergistically interacting components could be energetically less expensive, and partly because resistance to such a "multichemical" defense system would probably not arise so readily (Dolinger et al., 1973). Certainly, such arguments help rationalize the apparent dilemma of considerable material and energy resources being channeled into the synthesis of solanine even though this compound is biologically less effective than chaconine.

There is currently a growing realization that the outcome of plant–herbivore and plant–pathogen conflicts may be determined by the total biochemical profile of plant tissues rather than by single allelochemicals. The additional, and related, possibility that the biological/ecological impact of a particular biochemical environment may derive to a significant extent from interactions between component chemicals finds further support in the data presented here.

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